Supplemental information

FIGURE LEGENDS

Figure S1. Effects of fasting on GYS2 protein expression.

Wild-type and *Clock* mutant mice were maintained under a 12:12 h light-dark cycle (lights on at 0:00 and lights off at 12:00) and fasted overnight. Protein was extracted from the livers of these mice and levels of GYS2 protein were determined by Western blotting using anti-GYS2 antiserum. Effect of fasting on peak GYS2 protein levels (ZT 20). Open and closed bars, fed and fasted, respectively. Amount of protein was corrected relative to that of ACTIN. Maximal value for fasted wild-type mice is expressed as 100%. Values are means ± SEM (n = 3). Significant differences compared with values from fed wild-type mice are indicated as*$P < 0.05$. *P* values were calculated using Student's *t* test.

Figure S2. PPARα is not involved in circadian Gys2 expression.

Wild type and *Pparα* knockout mice were maintained under a 12:12 h light-dark cycle (lights on at 0:00 and lights off at 12:00). Total RNA was extracted from the livers of these mice and mRNA levels were quantified by real-time RT-PCR. Open and closed bars, wild-type and *Pparα* knockout mice, respectively. Amount of mRNA was corrected relative to that of *β-actin*. Maximal value for wild-type mice is expressed as 100%. Values are means ± SEM (n = 3).

Figure S3. Plasma insulin and glucagon levels

Mice were maintained under a 12:12 h light-dark cycle (lights on at 0:00 and lights off at 12:00). Open and closed symbols, wild-type and *Clock* mutant mice, respectively. Values are means ± SEM (n = 4). Significant differences compared with values from wild-type mice at each time point are indicated as $^{\dagger}P < 0.01$. *P* values were calculated using Student's *t* test.

EXPERIMENTAL PROCEDURES

Table S1. Cosinor analysis of Gys2 mRNA and protein expression and glycogen contents in liver of wild-type mice.

Circadian rhythms were statistically analyzed using the modified cosinor method. We defined the function as $f(x) = M+A\cos(2\pi/T(x-\varphi))$ and set 4 variables (M, mean statistics of rhythm [Mesor]; A, Amplitude [one-half of the total peak-trough variation]; T, period; φ, acrophase) as the fit parameters. Circadian period (T) was 24 hours under LD 12:12. The acrophase is expressed as delay from 0:00 in hours.

Figure S1. The effects of fasted condition on the GYS2 protein expression.

Mice were maintained under a 12:12 h light-dark cycle (lights on at 0:00 and lights off at 12:00) and fasted overnight. Mouse livers were homogenized in ice cold lysis buffer (5 mM Tris-HCl (pH 7.5), 15 mM NaCl, 0.1% Nonidet-40 and proteinase inhibitor cocktail (Roche Diagnostics K.K., Tokyo, Japan)). The lysates were boiled in 2× SDS sample buffer at 95°C for 5 min. Total protein (100 μg) was resolved by SDS-PAGE on 7.5% polyacrylamide gels, and then transferred onto PVDF membranes (GE healthcare Japan, Tokyo, Japan). Non-specific protein binding was blocked using 3% dried skim milk in PBS (17). Proteins on the membranes were immunoblotted against anti-GYS2 antiserum (gift from Banyu Pharmaceutical Co., Ltd.) and anti-ACTIN antibody (Nihon Millipore K.K, Tokyo, Japan). Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and ImmunoStar LD (Wako Pure Chemical Industries, Osaka, Japan). The amount of protein was corrected relative to that of ACTIN. The maximal value for wild-type mice is expressed as 100%. Values are described as means ± SEM (n = 3).

Figure S2. PPARα is not involved in circadian Gys2 expression.

We crossed *Pparα* knockout mice with Jcl:ICR mice. The resulting F1 pups that were heterozygous for the *Pparα* deletion were further mated to generate WT and *Pparα* KO littermates. Male *Pparα* KO and littermate WT mice aged 10–14 weeks were housed under a 12 h light–12 h dark cycle (lights on at 0:00 and lights off at 12:00) for at least 2 weeks before the day of the experiments. Total RNA was isolated from liver tissues

using RNAiso (TAKARA Bio Inc., Shiga, Japan) and then reverse-transcribed using the PrimeScript RT reagent kit (TAKARA Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The cDNA levels of genes of interest were measured by real-time quantitative PCR using a LightCycler (Roche Diagnostics K.K., Tokyo, Japan) with SYBR Premix Ex Taq (TAKARA Bio Inc., Shiga, Japan). Table 1 shows the sequences of the primer pairs. The amount of mRNA was corrected relative to that of *β-actin*. The maximal value for wild-type mice is expressed as 100%. Values are means ± SEM (n = 3).

Figure S3. Plasma insulin and glucagon levels

Male Jcl:ICR (Clea Japan Inc., Tokyo, Japan) and homozygous *Clock* mutant mice on a Jcl:ICR background at 7-10 weeks of age were maintained under a 12:12 h light-dark cycle (lights on at 0:00 and lights off at 12:00) for at least 2 weeks before the day of the experiments. Plasma insulin and glucagon was measured by ELISA with Insulin EIA (Shibayagi Co. Ltd., Gunma, Japan) and Glucagon EIA (Yanaihara Institute Inc., Shizuoka, Japan) kits according to the manufacturers' protocols.

Table S1.

Cosinor analysis of Gys2 mRNA and protein expression and glycogen contents in wild-type mouse liver.

	Acrophase (h)	Mesor	Amplitude
Gys2 mRNA	12.10 ±0.81	65.65 ±7.06	46.96 ±9.98
GYS2 protein	19.21 ±1.35	85.50 ±4.99	19.92 ±7.06
Glycogen	1.12 ±0.26	121.97 ±4.88	103.14 ±6.90

Acrophase, hours delay from 0:00 (light on); Mesor, mean statistics of rhythm; Amplitude, one-half the total peak-trough variation. Data are shown as means ±SEM.

Figure S1.

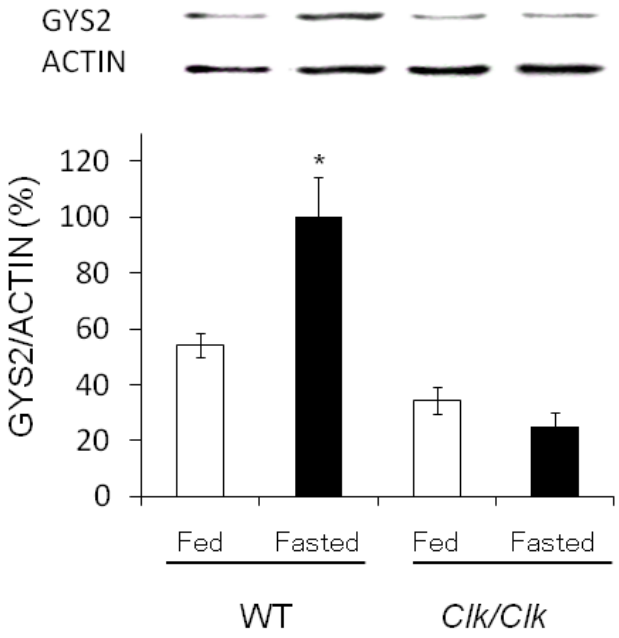

GYS2
ACTIN
GYS2/ACTIN (%)
120
100
80
60
40
20
0
*
Fed
Fasted
Fed
Fasted
WT
Clk/Clk

Figure S2.

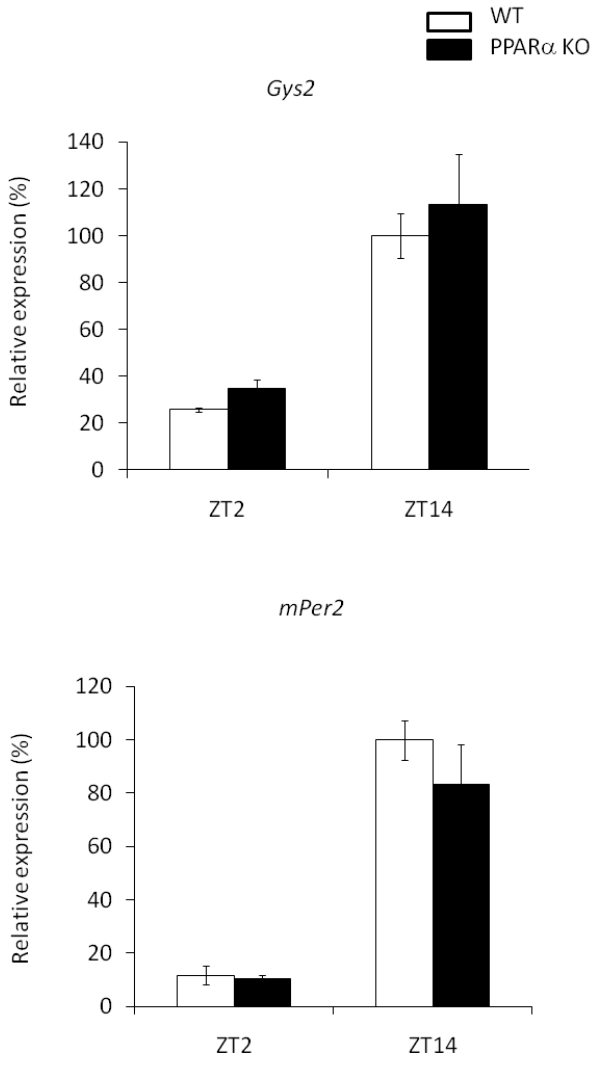
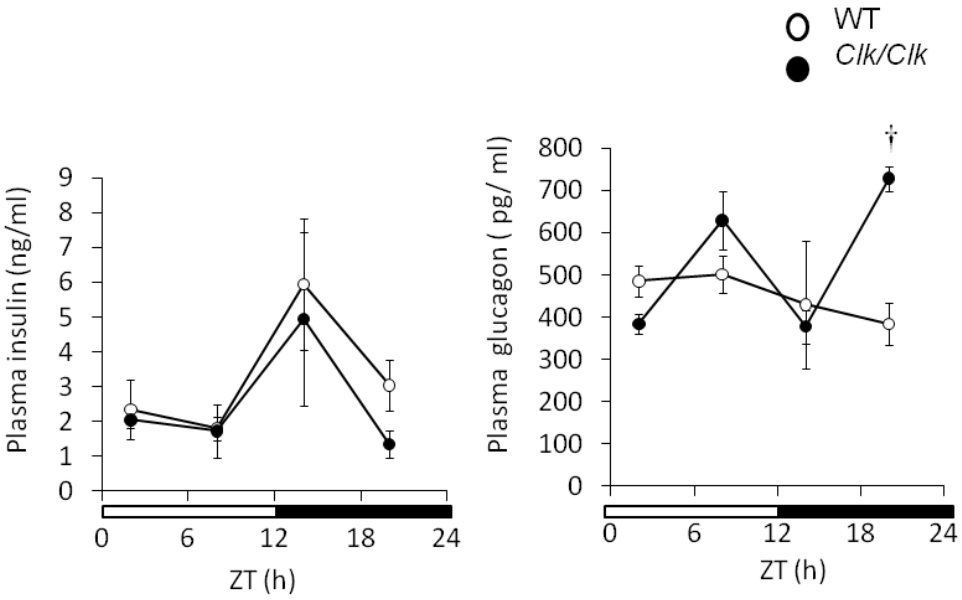

WT
PPARα KO
Gys2
Relative expression (%)
140
120
100
80
60
40
20
0
ZT2
ZT14
mPer2
Relative expression (%)
120
100
80
60
40
20
0
ZT2
ZT14

Figure S3.


WT
Clk/Clk
Plasma insulin (ng/ml)
0
1
2
3
4
5
6
7
8
9
0
6
12
18
24
ZT (h)
Plasma glucagon (pg/ ml)
0
100
200
300
400
500
600
700
800
†
0
6
12
18
24
ZT (h)